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Infectivity of *Cryptosporidium parvum* Oocysts after Storage of Experimentally Contaminated Apples

DUMITRU MACARISIN,[1] MÓNICA SANTÍN,[1] GARY BAUCHAN,[2] AND RONALD FAYER[1]*

[1]*U.S. Department of Agriculture, Agricultural Research Service, Beltsville Agricultural Research Center, Environmental Microbial & Food Safety Laboratory, 10300 Baltimore Avenue, Building 173, BARC-East, Beltsville, Maryland 20705; and* [2]*U.S. Department of Agriculture, Agricultural Research Service, Electron & Confocal Microscopy Unit, 10300 Baltimore Avenue, Building 465, BARC-East, Beltsville, Maryland 20705, USA*

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ABSTRACT

Irrigation water and washing water have been inferred to be associated with contamination of fresh fruits and vegetables with pathogenic microorganisms infectious for humans. The objective of the present study was to determine whether apples experimentally contaminated with *Cryptosporidium* oocysts represent a food safety concern. Laser scanning confocal microscopy revealed no morphological changes in *Cryptosporidium parvum* oocysts attached to apples after 6 weeks of cold storage, suggesting that oocysts might remain viable and possibly infectious during prolonged storage. Mice were fed apple peels from experimentally contaminated apples to determine whether oocysts had remained infectious on apples stored for 4 weeks. All mice developed cryptosporidiosis. To evaluate the strength of oocyst attachment to apples, washing methods that have been reported to be helpful for recovery of oocysts from various foodstuffs were evaluated, except that the intensity of washing was increased in the present study. None of the tested washing methods succeeded in completely removing oocysts from the apple peel. The most efficient removal (37.5%) was achieved by rigorous manual washing in water with a detergent and by agitation in an orbital shaker with Tris–sodium dodecyl sulfate buffer. Glycine and phosphate-buffered saline buffers had no effect on oocyst removal. Scanning electron microscopy revealed that some oocysts were attached in deep natural crevices in the apple exocarp and others were attached to the smooth surface of the peel. Some oocysts were closely associated with what appeared to be an amorphous substance with which they might have been attached to the apple surface.

Unpasteurized fresh apple juice, also known as apple cider, has been related to foodborne outbreaks of cryptosporidiosis *(1, 3, 9, 16)*. Some of these outbreaks have been associated with the presence of cattle in the orchards and the lack of good manufacturing practices in the cider-pressing facilities *(16)*. Apples picked from the ground, sometimes referred to as ''drops,'' ''grounders,'' or ''windfalls,'' were suggested as a principal source of cider contamination with pathogenic microorganisms *(12)*. However, an extensive study conducted at five major producers of unpasteurized apple cider in Ontario, Canada, revealed no correlation between fruit quality or harvest technique and the presence of *Cryptosporidium* oocysts in the resulting cider *(9)*. In this study, *Cryptosporidium* oocysts were found in the water (including well and municipal drinking water) used to clean apples and on processing equipment, suggesting that water could be a potential source of contamination.

Cryptosporidium oocysts have been found in 87% of the surface waters and in 3.8 to 40% of treated drinking water in the United States *(13, 14, 21)*. Apple orchards are subject to intensive spraying for pest control and plant nutrition *(19)*. For bearing apple orchards, the total number of sprayings ranges from 9 to 15 per growing season. In some geographic regions, apple orchards also require frequent irrigation *(24)*. Sprinkler irrigation of apple trees is optimal for fruit growth *(10)*. More than 85% of the modern irrigated apple orchards in the Pacific Northwest use sprinkler irrigation, and widespread adaptation of over-tree sprinkling is used because of economic benefits of the evaporative cooling of apples, which increases fruit color, improves storage life after harvest, prevents sun scald and sun burn, and increases total photosynthesis *(7, 8, 11, 26)*. To cover spraying and irrigation needs, especially during periods of drought, growers might use open surface water from ponds, lakes, and streams, which are frequently exposed to wildlife and livestock. Studies on the potential sources of crop contamination with human pathogenic parasites suggested that irrigation waters could be a major route of contamination for fresh produce *(4, 20, 22, 25)*.

Because *Cryptosporidium* oocysts have been found in irrigation water *(25)* and because experiments have revealed firm adherence of oocysts to irrigated spinach leaves *(15)*, we hypothesized that apples also could become contaminated with *Cryptosporidium* oocysts as a result of orchard irrigation and spraying with contaminated water. The objective of the current study was to determine whether oocysts of *Cryptosporidium parvum*, a waterborne pathogen, could adhere to experimentally contaminated apples and remain infectious during postharvest storage.

* Author for correspondence. Tel: 301-504-8750; Fax: 301-504-6608; E-mail: ronald.fayer@ars.usda.gov.

MATERIALS AND METHODS

Source of *C. parvum* oocysts. *C. parvum* (Beltsville isolate) oocysts were isolated from feces of an experimentally infected calf and concentrated using a CsCl protocol as described by Santín et al. *(23)*.

Experimental contamination and storage of apples. Golden delicious apples were handpicked at full maturity in an experimental orchard at the Appalachian Fruit Research Station (Kearneysville, WV). Freshly harvested apples were rinsed using warm tap water and dried on paper towels at room temperature. Apples were then transferred to plastic trays and compactly arranged to avoid fruit rolling. About 5,000 *C. parvum* oocysts suspended in water were pipetted onto four separate sites on the surface of each apple. Every contaminated site was outlined with a permanent marker. To study the persistence of oocysts on apples, 80 apples were contaminated. For the infectivity assays, 30 apples were contaminated at 4 weekly intervals. One hour after contamination, apples were transferred to a refrigerator and stored at 6°C.

LSCM analysis of *C. parvum* oocysts on apples. At 2, 3, and 6 weeks after contamination with *C. parvum* oocysts, contaminated spots on the apple surface were examined by laser scanning confocal microscopy (LSCM). Apples were removed from cold storage and held at room temperature for 1 h. Ten microliters of MeriFluor solution (Meridian Bioscience, Cincinnati, OH) was applied to each contaminated area and incubated for 15 min at room temperature in the dark. Five milliliters of 50 mM Tris–sodium dodecyl sulfate (SDS) buffer was used to remove any unbound antibody. Excess buffer was removed by pipetting sterile water directly onto each contamination site. Thin layers of tissue within the marked area were peeled from the fruit surface with a razor blade. Peels were then placed in petri dishes (MatTeck Corp., Ashland, MA) with cover glass bottoms and immersed in a biological buffer (10 mM 2-(*N*-morpholino) ethanesulfonic acid, pH 6.5) for microscopic examination. A Zeiss 710 LSCM system was utilized (Zeiss, Thornwood, NY). The images were observed with a Zeiss Axio Observer inverted microscope with a water immersion objective (40 by 1.2 NA) and an oil immersion planapochromatic objective (63 by 1.4 NA). A photomultiplier tube captured the light emitted from a 488-nm argon laser with a pin hole of 3.7 μm passing through a MBS 488 filter with limits set at 492 to 543 nm for detection of fluorescein and 647 to 721 nm for detection of autofluorescence from chloroplasts. Zeiss Zen 2008 software was used to obtain the images.

Mouse infectivity assays. A mouse infectivity assay was conducted to determine whether *C. parvum* oocysts attached to the surface of apples could remain infectious after storage. Neonatal CD-1/ICR mice (5 to 7 days old) were purchased from the National Institute of Health (Bethesda, MD). Mice were allowed to acclimate for 4 days and then were immunosuppressed by adding 0.005% dexamethasone (vol/vol) to their drinking water for 5 days. Apples stored for 1, 2, 3, and 4 weeks after contamination were used in the infectivity assays. Thin slices of apple peel from the contaminated areas were mixed with normal rodent chow at a ratio of 9:1 (wt/wt) and fed to three mice for each storage time period. For positive controls, slices of apple peel were contaminated with fresh *C. parvum* oocysts just before being fed to mice. Immunosuppressed mice that received peels from noncontaminated apples were considered negative controls. Fecal samples were collected every day from each individual mouse from day 3 to day 7 postfeeding. Fecal samples were checked for *Cryptosporidium* oocysts by fluorescence microscopy and MeriFluor solution. On the day 7, the terminal ileum was removed from each mouse at necropsy. DNA was extracted from ileum segments as described below for apple peels, except that only 20 μl of proteinase K per sample was used followed by an overnight incubation. A nested PCR assay was conducted as described below.

All positive PCR products from ileum samples were purified using exonuclease I–shrimp alkaline phosphatase (USB Corporation, Cleveland, OH) and sequenced in both directions with the same PCR primers as used in the secondary PCR in 10-μl reactions, Big Dye chemistries, and an ABI 3100 sequencer analyzer (Applied Biosystems, Foster City, CA). Sequence chromatograms of each strand were aligned and examined with Lasergene software (DNASTAR, Inc., Madison, WI).

Evaluation of the persistence of *C. parvum* on apples. The effectiveness of sonication, orbital shaking, and manual washing for removal of oocysts from the surface of the apples was compared. Sonication and orbital shaking were performed in three different dissociating buffers. Two of these buffers have been used before on fresh produce contaminated with protozoan parasites; buffer 1 was 1 M glycine (pH 5.5) *(5)* and buffer 2 was 1× phosphate-buffered saline (PBS, pH 7. 2) with 0.01% (vol/vol) polysorbate surfactant, Tween 20 *(2)*. Buffer 3 was 10 mM Tris HCl (pH 7.5) with 0.1% SDS. Contaminated apples were removed from cold storage and rinsed under flowing warm water. Eight to 10 contaminated apples were used for each treatment. Apples were placed in 3 2-liter glass beakers, each containing 1 liter of each of the dissociating buffers, and agitated at 100 rpm for 15 min using an orbital shaker. The wash buffer was discarded, and apples were rinsed with flowing tap water. For sonication, contaminated apples were placed in an ultrasonic cleaner (Mettler Electronics Corporation, Anaheim, CA), 1 liter of each dissociating buffer was added, and the sample was sonicated twice for 1 min each time with manual reposition of the apples between treatment cycles. The wash buffer was discarded, and apples were rinsed with flowing tap water. For manual washing, apples were rigorously washed by hand with warm water and Dial antibacterial liquid soap (0.2% Triclosan active ingredient). After all washing procedures, apple surfaces were wiped with Kimwipes (Kimberly-Clark, Dallas, TX).

Contaminated areas from each apple were peeled with a sterile surgical blade and used for assay of oocysts on apples. Total DNA was extracted using a DNeasyTissue Kit (Qiagen, Valencia, CA) with a slightly modified protocol. Approximately 15 to 30 mg of peel from each apple was suspended in 180 μl of buffer ATL, and 20 μl of proteinase K (20 mg/ml) was added and thoroughly mixed by vortexing. After 4 h of incubation, another 20 μl of proteinase K was added to each tube, and tubes were incubated at 56°C overnight. The remaining protocol followed the manufacturer's instructions except that the nucleic acid was eluted in 100 μl of buffer AE to increase the quantity of recovered DNA. Positive controls were represented by peels from unwashed contaminated apples. Negative controls were peels from noncontaminated apples.

A two-step nested PCR protocol was used to amplify an 830-bp fragment of the small subunit rRNA gene with primers 5′-TTCTAGAGCTAATACATGCG-3′ and 5′-CCCATTTCCTTCGAAACAGGA-3′ for the primary PCR and 5′-GGAAGGGTTGTATTTATTAGATAAAG-3′ and 5′-AAGGAGTAAGGAACAACCTCCA-3′ for the secondary PCR *(27)*. The primary PCR mixture contained 1× PCR buffer, 3 mM $MgCl_2$, 0.2 mM concentration of deoxynucleoside triphosphates, 2.5 U of *Taq* polymerase (Qbiogene, Irvine, CA), 2.5 μl of bovine serum albumin (0.1 g/10 ml), and 1 μM concentrations of each forward and reverse primer in a 50-μl reaction volume. PCRs were processed with an initial hot start at 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 59°C for 45 s, and 72°C for 1 min, and

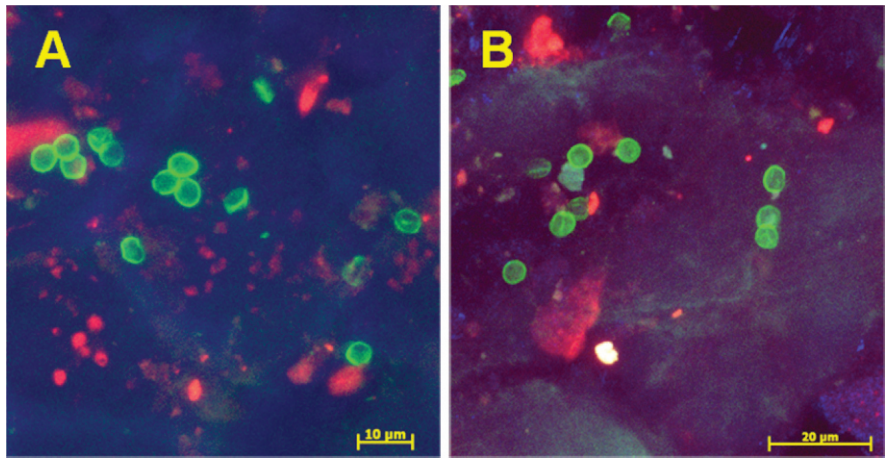

FIGURE 1. *Visualization of* Cryptosporidium *oocysts by a laser scanning confocal microscopy on apples with fluorescein-labeled monoclonal antibodies. Multichannel confocal images of* C. parvum *oocysts (green fluorescence) attached to apple surface at 2 weeks (A) and 6 weeks (B) after contamination.*

ending with 72°C for 7 min. The secondary PCR mixture was identical except that the $MgCl_2$ concentration was 1.5 mM. The processing program was an initial hot start at 94°C for 3 min, 40 cycles of 94°C for 30 s, 58°C for 90 s, and 72°C for 2 min, and a final extension at 72°C for 7 min. A positive genomic DNA control (*C. parvum*) and negative control (distilled water) were included with each PCR run. PCR products were analyzed on 1% agarose gel and visualized after ethidium bromide staining. All negative samples were reprocessed spiked with genomic control DNA at a concentration equal to that used in the positive control to determine whether the sample was indeed negative or whether there was an inhibitor in the sample.

The number of samples generating positive signal, as detected by PCR, was calculated and expressed as the percentage from the total number of samples (ranging from 32 to 40). The postwashing percentage of positive samples represented the degree of persistence of *C. parvum* oocysts on apples.

LT-SEM analysis of *C. parvum* attachment to apples. Enhanced visualization of oocysts on apples was conducted by low-temperature scanning electron microscopy (LT-SEM) with an S-4700 field emission scanning electron microscope (Hitachi High Technologies America, Inc., Pleasanton, CA) equipped with a Polaron Polar Prep 2000 cryotransfer system (Energy Bean Sciences, East Grandby, CT). Apple peels contaminated with *Cryptosporidium* were placed on copper plates (16 by 30 mm) that contained a thin layer of Tissue Tek (OCT Compound, Ted Pella, Inc., Redding, CA) and were flash frozen by placing the plates on a precooled (−96°C) brass bar whose lower half was submerged in liquid nitrogen. Frozen samples were transferred to a liquid nitrogen Dewar for future use or cryotransferred under vacuum to the cold stage in the prechamber of the cryotransfer system. Removal of any surface contamination (condensed water vapor) took place in the cryotransfer system by etching the frozen specimens for 10 to 15 min by raising the temperature of the stage to −90°C. After etching, the temperature was lowered below −130°C, and a magnetron sputter head equipped with a platinum target was used to coat the specimens with a very fine layer of platinum. The specimens were transferred to a precooled (−140°C) cryostage inside the scanning electron microscope for observation. An accelerating voltage of 10 kV was used to view the specimens. Images took 80 s to digitize at a capture resolution of 2,560 by 1,920, with a resulting 4.8 M image. Images were sized and placed together with Photoshop 7.0 (Adobe Systems, San Jose, CA) to produce a single multipanel figure.

RESULTS

LSCM analysis of *C. parvum* oocysts on apples. LSCM analysis of contaminated apples indicated that *C. parvum* oocysts were attached to the surface (Fig. 1). No morphological changes in oocysts were found after prolonged cold storage (up to 6 weeks) (Fig. 1B), suggesting that oocysts that adhered to apples can remain viable and possibly infectious during storage.

Assessment of the infectivity of *C. parvum* oocysts attached to apples. *Cryptosporidium* was detected by PCR in the ileum of all mice fed peels from apples contaminated and stored for 1 to 4 weeks and in all mice fed peels from freshly contaminated apples. Mice fed noncontaminated apple peels did not become infected.

Evaluation of the persistence of *C. parvum* oocysts on apples. The most efficient methods for eliminating oocysts were rigorous manual washing in water with liquid soap and orbital shaking with buffer 3 (Tris-SDS). Both methods reduced contamination of the 40 peel disks (4 disks per apple) to 62.5% (Fig. 2). Buffers 1 and 2 were less efficient for removing oocysts from apples, and all peel samples from apples washed with these buffers were still contaminated with the parasite after washing.

LT-SEM analysis of *C. parvum* attachment to apples. LT-SEM analysis of *C. parvum* oocysts on apple surfaces was conducted to elucidate the strong persistence of oocysts on apples. Classical SEM preparation techniques involving immersion fixation, dehydration, and critical point drying cause significant deleterious changes in specimen structure due to effects of surface tension, osmotic stress, and mechanical damage. LT-SEM allows significantly higher magnification with a greater depth of field without dissolving structures of interest or denaturing the sample. Some oocysts were embedded in naturally occurring crevices in the apple peel (Fig. 3A through 3D) and others were attached to smooth areas of the apple peel (Fig. 3E and 3F). Oocysts on the relatively smooth areas of the peel were adjacent to an amorphous extracellular matrix with which they appeared to be attached to the apple surface (Fig. 4).

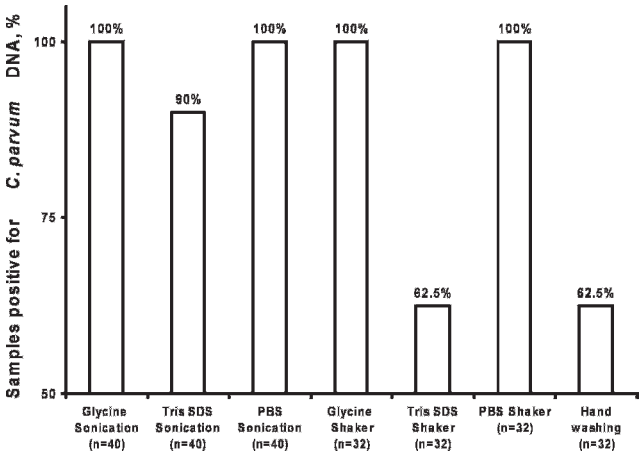

FIGURE 2. *Removal of oocysts from contaminated apples by orbital shaking at 100 rpm for 15 min and sonication (two cycles of 1 min each) in glycine, Tris-SDS, and PBS buffers, and by manual washing. Histogram shows the percentages of persisting contamination in apples after washing procedures as determined by the presence of* C. parvum *DNA.*

DISCUSSION

Under the experimental conditions in the present study, *Cryptosporidium* oocysts adhered to apples. LSCM indicated that the majority of *Cryptosporidium* oocysts on experimentally contaminated apples stored in a cold chamber at an average temperature of 6°C (conditions characteristic for fruit storage houses) appeared to be normal with no morphological changes as a result of dehydration for up to 6 weeks after contamination (Fig. 1). This finding suggests that under commercial postharvest conditions oocysts can remain viable and potentially infectious on fresh fruits for several weeks. The infectivity assays with neonatal mice revealed that *C. parvum* oocysts remained infectious on apples stored for as long as 4 weeks. All immunosuppressed mice became infected after eating peel from contaminated apples The presence of *Cryptosporidium* oocysts was confirmed by fluorescence microscopy, molecular analysis of animal feces, and detection of *Cryptosporidium* in tissues from the terminal ileum by PCR assay. All *Cryptosporidium*-positive PCR products were sequenced to confirm that the infectious agent was the *C. parvum* isolate used to infect the mice and not another *Cryptosporidium* species or genotype.

The presence of infectious oocysts on storage apples stimulated interest in determining the strength of oocyst attachment to the apple surface. Thus, to evaluate the persistence of the parasite on apples, three different washing techniques for removing the oocysts from the fruit were compared. Selection of the elution techniques partially relied on previous scientific reports evaluating the efficiency of recovery of *C. parvum* oocysts from various foodstuffs *(2, 5)*. The objective of the current study was to completely

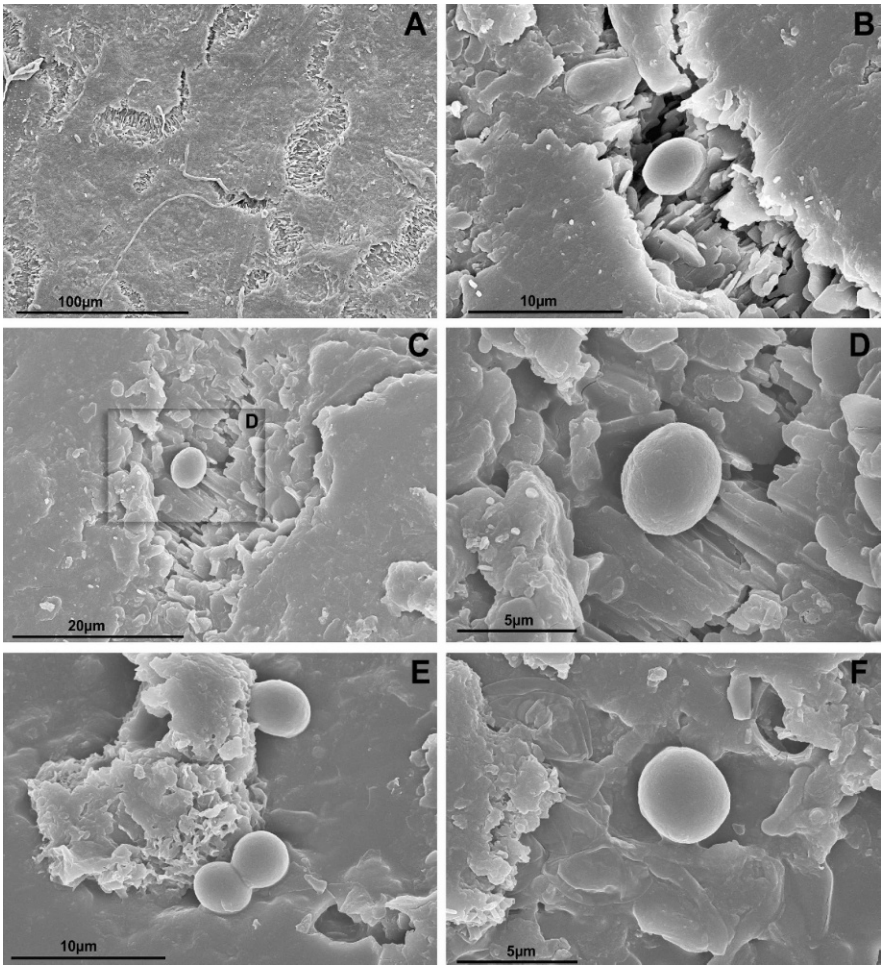

FIGURE 3. *Low-temperature scanning electron microscopy images of* C. parvum *oocysts on apple surface. (A) Fruit surface showing naturally occurring multiple crevices in the peel. (B through D)* C. parvum *oocysts embedded in crevices of the apple peel. (E and F) Parasite oocysts attached to smooth areas of the apple.*

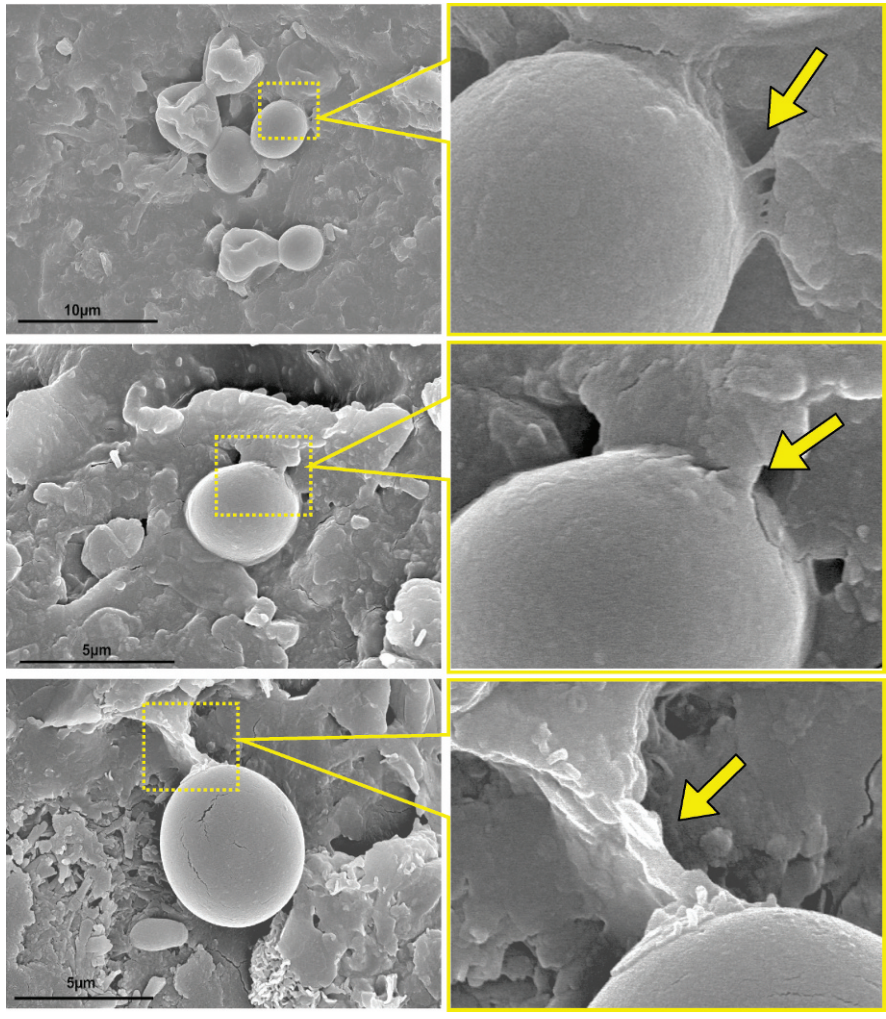

FIGURE 4. *Low-temperature scanning electron microscopy images of* C. parvum *oocysts attached to smooth areas of the apple peel. Right panels: in magnified images all oocysts have an amorphous extracellular matrix with which they appear to be attached to fruit surface (yellow arrows).*

remove the oocysts from the apple surface without regard to the recovery rates; therefore, the intensity of washing was greatly increased compared with those methods evaluated previously. Orbital shaking was conducted at 100 rpm for 15 min rather than at 80 rpm for 1 min as reported by Cook et al. *(5)*. Of the physical methods for extracting oocysts from fresh produce, Cook et al. *(5)* found that pulsification yielded the highest recovery of oocysts. Because pulsification of apples in a stomacher bag would homogenize the fruit, making further detection of *C. parvum* oocysts on contaminated areas of the surface impossible, sonication was used in the present study. Two sonication cycles of 1 min each were used rather the single 1-min pulsification cycle used by Cook et al. *(5)*. Although tested buffers differed in their elution efficiency, all failed to completely remove oocysts from apples. A relatively high percentage of the persisting contamination, as determined from the presence of *Cryptosporidium* DNA, was detected in the majority of samples after all washing treatments (Fig. 2). When Garcia et al. *(9)* investigated sources of microbial contamination in apple cider, *Cryptosporidium* was not detected in the eluates; however, the cider pressed from the same fruit batches was positive for the parasite. Garcia et al. *(9)* used plain water to elute *Cryptosporidium* oocysts from harvested fruits, which may explain why *Cryptosporidium* was not detected on apples before pressing. The current study indicated that oocysts are highly resistant to elution and are firmly attached to the apple peel. LT-SEM analysis of contaminated peel areas revealed oocysts in exocarp crevices and attached directly to the waxy cuticle of the peel (Fig. 3). Oocysts observed on peel epidermis were surrounded or even covered by an extracellular matrix that appeared to allow the oocysts to adhere to the apples (Fig. 4). Few experimental data have been published regarding the external layer of the *Cryptosporidium* oocyst wall; this wall is labile to bleach used to remove contaminating bacteria from the parasite and therefore usually cannot be found during microscopic observations *(18)*. Experimental evidence suggests that the extracellular matrix, also referred to as an outer veil, covering *C. parvum* oocysts is of a glycoprotein nature *(17)*. Nanduri et al. *(17)* separated the surface coat of the oocyst and subjected it to carbohydrate composition analysis, which revealed the high similarity with glycocalyx material. The role of the microbial glycocalyx in adhesion to host cells, biofilm formation, and resistance to environmental stressors is well documented *(6)*. Similarly, highly adhesive carbohydrate moieties might cover the outermost layer of the oocyst wall (glycocalyx) and participate in the strong adhesion of oocysts to fruits and vegetables. A better understanding of the biochemical nature of this extracellular matrix will be useful for developing efficient methods for removing

oocysts from food matrices and will result in more accurate detection of oocysts in fresh produce.

In conclusion, the current study revealed that under experimental conditions *C. parvum* oocysts can strongly attach to the surface of apples and can remain infectious for about 1 month after the contamination event. Whether contaminated apples are meant for fresh consumption or for cider production, they can serve as a potential source of foodborne cryptosporidiosis. Spraying or irrigating apple orchards with contaminated water might result in fruit contamination. Field studies are needed to assess the likelihood of this type of contamination under natural conditions.

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